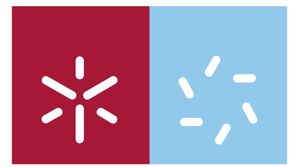

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Tese de Mestrado
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Trabalho efectuado sob a orientação da
Doutora Fátima Baltazar

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ABSTRACT/RESUMO

ABSTRACT

According to the literature, the human embryo and human placenta develop in a hypoxic environment during the first trimester. In this way, cells have to resource to glycolitic pathways for energy supply, leading to an intracellular accumulation of monocarboxylates such as lactate and pyruvate. These acids have an important role in cell metabolism and their rapid transport across the plasma membrane is crucial for the maintenance of intracellular pH homeostasis. This transport is mediated by a family of transporters, designated as monocarboxylate transporters (MCTs), namely isoforms 1, 2, 3 and 4. Regarding the regulation of MCTs, it is known that MCT1, MCT3 and MCT4 depend on an ancilary protein, CD147, while MCT2 depends on GP70 for activity and correct localization in the plasma membrane. Their expression is also regulated by hypoxia, being HIF-1α an important regulator of MCT4 expression.

The general aim of this project was to characterize the expression of MCT1-MCT4 during lung embryo development of Rattus Norvegicus and over several stages of embryo development of Gallus gallus to elucidate the function of MCTs in embryogenesis. Furthermore, we intended to study the role of MCTs by studying the effects of inhibition their expression in fetal lung explants culture.

Our findings identified the expression pattern of MCT4 during chick embryo development.

Regarding rat lung embryonic development, our results showed that the expression of MCTs varies during lung embryonic development and that CHC (α-cyano-4-hydroxycinnamate) appears to have an inhibitory effect on lung branching and viability in a dose dependent way.

To conclude it appears that MCTs play a role in embryo development, since their expression varies along embryogenesis. Lung explant and viability seemed to be sensitive to MCT inhibition, however, to prove the dependence of MCT activity further studies will be needed.

RESUMO

Segundo a literatura, o embrião humano e a placenta desenvolvem-se num ambiente de hipóxia durante o 1º trimestre de gestação. Deste modo, as células recorrem as vias glicolíticas para a obtenção de energia, resultando num acúmulo intracelular de monocarboxilatos, como o lactato e o piruvato. Estes têm um papel fundamental no metabolismo celular e o seu rápido transporte através da membrana é vital para a manutenção do pH da célula. Este transporte é mediado por proteínas transmembranares conhecidas por transportadores de monocarboxilatos (MCTs), nomeadamente as isoformas 1, 2, 3 e 4. Relativamente à regulação da expressão dos MCT1 e MCT4, sabe-se que necessitam de uma proteína auxiliar, CD147, para correcta expressão membranar e actividade. A sua expressão é também regulada pela hipóxia, sendo o factor de transcrição HIF-1α um importante regulador da expressão do MCT4.

O objectivo geral desta tese foi caracterizar a expressão dos MCT1-MCT4 durante o desenvolvimento embrionário do pulmão de *Rattus norvegicus* e ao longo de vários estadios do desenvolvimento embrionário de *Gallus gallus* de modo a elucidar a função dos MCTs na embriogénese. Além disso estudou-se o papel dos MCTs através da inibição da sua actividade em cultura de explantes de pulmão de rato.

Neste trabalho descrevemos o padrão de expressão do MCT4 durante o desenvolvimento embrionário de galinha.

No que diz respeito ao desenvolvimento embrionário de pulmão de rato, os nossos resultados mostraram que a expressão dos MCTs varia ao longo do desenvolvimento embrionário do pulmão e que o CHC (α-cyano-4-hydroxycinnamate) parece ter um efeito inibitório na ramificação do pulmão e da viabilidade de uma maneira dose dependente.

Para concluir, parece que os MCTs desempenham um papel no desenvolvimento do embrião, uma vez que a sua expressão varia ao longo embriogênese. Explantes de pulmão e a sua viabilidade pareceu ser sensível à inibição dos MCTs, no entanto, estudos adicionais serão necessários para provar a dependência na atividade dos MCT.

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ABBREVIATIONS

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CHC: α-cyano-4-hydroxycinnamate;

DIDS: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid;

Dpc: day pos-conception;

EtBr: Ethidium bromide

GD: gestational day;

HH: Hamburger-Hamilton;

HIF-1: hypoxia inducible factor-1;

IHC: Immunohistochemistry;

ISH: *In situ* hybridization;

LDH: lactate dehydrogenase;

MCT: H+-monocarboxylate cotransporter;

MMP: matrix metalloproteinase;

pCMBS: p-chloromercuribenzenesulfonic acid;

pH_i: intracellular pH;

RPE: Retinal pigment epithelium;

RT: Room temperature;

TM: transmembrane α-helical;

VEGF: vascular endothelial growth factor;

CHAPTER 1 – GENERAL INTRODUCTION

Developmental biology

The process by which the embryo is formed and develops is known as embryogenesis. It is initiated by a process called fertilization that is the fusion of genetic material from the two gametes, the sperm and the egg. After that cleavage begins which consist on a series of rapid mitotic divisions wherein the zygote is divided into numerous smaller cells. These different cells derived from cleavage, up to the blastula stage, are called blastomeres. The blastula is usually a spherical layer of cells, the blastoderm, surrounding a fluid-filled or yolk-filled cavity, the blastocoel. The cells then migrate to the interior of the blastula, consequently forming three germ layers. During this process the embryo is called gastrula. The germ layers are referred to as the ectoderm, mesoderm and endoderm and give rise to all of an animal's tissues and organs through the process of organogenesis [1].

Embryogenesis: cellular metabolism

Embryonic and fetal development is strongly influenced by the oxygen environment [2, 3].

Studies relative to placenta formation indicate that the human embryo and human placenta are developed in a hypoxic environment during the first trimester. The placenta consists of a gestational sac, villi with few capillaries, a trophoblast layer and an exocoelomic cavity which do not contain oxygen transport system but anti-oxidant molecules are present, protecting the embryo from oxidative damage. Thus, these features are indirect evidence that the gestational sac limits the embryonic oxygen exposure [4, 5]. Low oxygen concentrations induce a whole spectrum of cellular and systemic responses [6, 7]. The oxygen level in the cells and organs is regulated by pathways that affect the expression and activity of numerous cellular proteins [8]. Sensing and responding alterations in oxygen tension are important variations in the physiology, and tissues have developed essential mechanisms of response to physiological oxygen reduction [4].

Hypoxia is commonly associated with pathologies such as tissue ischaemia and inflammation. However, hypoxic microenvironments also occur in both the developing embryo and the adult, and often create specific niches that regulate cellular differentiation [9, 10]. Molecular mechanisms through which O_2 levels interfere with embryonic development have been elucidated by the characterization of hypoxia inducible factors (HIFs), which are dimeric transcription factors that regulate hypoxic responses in cells and tissues [8, 11, 11]. HIF-1 is considered a master switch that allows cells to respond to

falling oxygen levels. The protein consists of two essential subunits, HIF-1α and HIF-1β, which heterodimerize [12]. Many genes are transcriptionally activated by HIF-1 in response to hypoxia, such as glucose transporters, glycolitic enzymes, and vascular endothelial growth factor (VEGF) for angiogenesis [2, 3]. Some authors suggest that the initial low hypoxia levels normally present within the heart fields and neural tissue activate HIF1-inducible genes for normal development [4]. The fundamental effects of oxygen levels on development, physiology, and disease pathophysiology are now being recognized. It seems that the development takes place in a physiologically low oxygen environment and energy demands rely on glycolysis [4, 13]. Preimplantation embryos *in vivo* develop in uterine fluid and they derive their ATP predominantly by oxidative metabolism of pyruvate, lactate, and amino acids. The mouse embryo becomes more dependent on aerobic glycolysis after implantation [13]. Mouse embryos (gestational day 6.5–9.5) grown *in vitro* convert 90% of the catabolized glucose to lactate, even though the embryos are cultured in 20% oxygen, which should favor oxidative pathways [14]. For rat and mouse embryos, it appears that for the start of the organogenic period, including the period of neural tube closure, the embryo is largely dependent on glycolysis even when conditions favor more efficient pathways [15, 16]. However, the availability of oxygen to the embryo *in vivo* is not really known [3]. During the early stages of human and mouse, pyruvate and lactate are the most important energy sources. Pyruvate, besides the function of energy production, has ability to react with hydrogen peroxide [17] and / or may serve as a means of removing ammonia of the embryo, converting it into alanine [18]. Lactate formed by early human embryos derived from pyruvate [19] and its exchange with the extracellular space, seems to be important for the regulation of the intracellular pH (pHi) and / or to maintain the NAD/NADH ratio [20].

Cellular function depends on its metabolism, and metabolism generates intracellular acid by the formation of H^+ ions. The ability of cells to control their ionic composition in response to external ionic stress is, therefore, likely to be an important determinant of developmental progress, but there are limited knowledge on the ion transporters and channels at these early stages of development. For example, H^+ ions can exert particularly powerful influences on cell function, and so understanding the nature and properties of the proton transport and buffering systems available to the conceptus is of particular significance[21, 22]. The pHi may also play a role in developmental signaling. It has been described as an important trigger for the later developmental events of neural induction [23, 24] and posterior axial [24]. The importance of the pHi for

successful early development was first demonstrated empirically in Bavister's studies on the pH dependence of monospermic fertilization in the hamster. Cells regulate pHi through exchangers on the plasma membrane. These transports include Na^+/H^+ exchanger, Na^+,HCO_3^-/CL^- exchanger, however, the mechanism of pHi regulation during embryo development is not really elucidated [25].

Posterior studies were done and demonstrated that controlling pHi could be also carried out by a family of transporters designated by H+-monocarboxylate cotransporter (MCT) [25].

Metabolism and Monocarboxylate transporters

Monocarboxylic acids such as lactate and pyruvate play an important role in cellular metabolism. Some tissues like white skeletal muscle, erythrocytes and many tumour cells depend on glycolysis to produce their ATP under normal physiologic conditions, while all tissues become dependent on glycolysis during conditions as hypoxia and ischemia [26].

Human cells have a series of mechanism in order to generate and meet the primary energy demands of cell metabolism. One of the mechanisms is glycolitic pathway, which involves a series of cytoplasmatic enzymes that converts glucose into pyruvate [27]. Then, pyruvate enters the mitochondria for the realization of oxidative phosphorylation. These two processes in sequences generate the equivalent of 38 ATP units per glucose molecule [27, 28]. Such is the energetic fate of glucose in healthy tissue under normal physiological conditions. Under anaerobic conditions, where oxygen is limited, glycolysis may be the main energy source of an organism. More specifically, the differences in aerobic and anaerobic respiration depend on the different roles played by the NADH molecules produced. In both aerobic and anaerobic respiration, NADH is an enzyme complex and must be restored to its oxidized state (NAD). Under aerobic conditions, meaning oxygen availability, NADH can be transported into the mitochondria where it can be immediately reoxidized NAD, playing a role in the electron transport chain. However, under anaerobic conditions, NADH is reoxidized to NAD through anaerobic mechanisms, either homolactic or alcoholic fermentation. Pyruvate, product of glycolysis, is converted into lactic acid by the enzyme lactate dehydrogenase (LDH). In this reaction, the hydrogen from NADH molecule is transferred to the pyruvate molecule, leading to production of lactate. From the lactate product, lactic acid can be formed. These anaerobic conditions lead to glycolytic products other than pyruvate. These different

products are necessary for NADH reoxidization so that it allows in the next round of glycolysis [29]. Lactic acid transport across the plasma membrane is fundamental for metabolism and pH regulation of all cells, by removing lactic acid produced by glycolysis and allowing uptake by those cells that utilize it as a respiratory fuel, as does the heart [30]. If lactic acid effluxes do not happen, the intracellular concentration increase leads to a decrease in pH inside the cell, leading to the inhibition of glycolysis [31]. Although it is lactic acid that is both produced and utilized by metabolism, the p*K* of lactic acid is 3.86, which ensures that it dissociates almost entirely to the lactate anion at physiological pH. This charged species cannot cross the plasma membrane readily by free diffusion, but require a specific transport mechanism, provided by MCTs [26, 30]. Transport of monocarboxylates via MCTs is fully reversible and the direction of transport is determined by both substrate and proton gradient [20].

Although lactate is the monocarboxylate which transport through the plasma membrane takes place in highest quantities, MCTs are also essential for the transport of other metabolically important monocarboxylates such as pyruvate and ketone bodies. So being, MCTs have a central role in mammalian metabolism and are critical for the metabolic communication between cells [26].

Figure 1 shows the metabolic pathways involved in monocarboxylate transport.

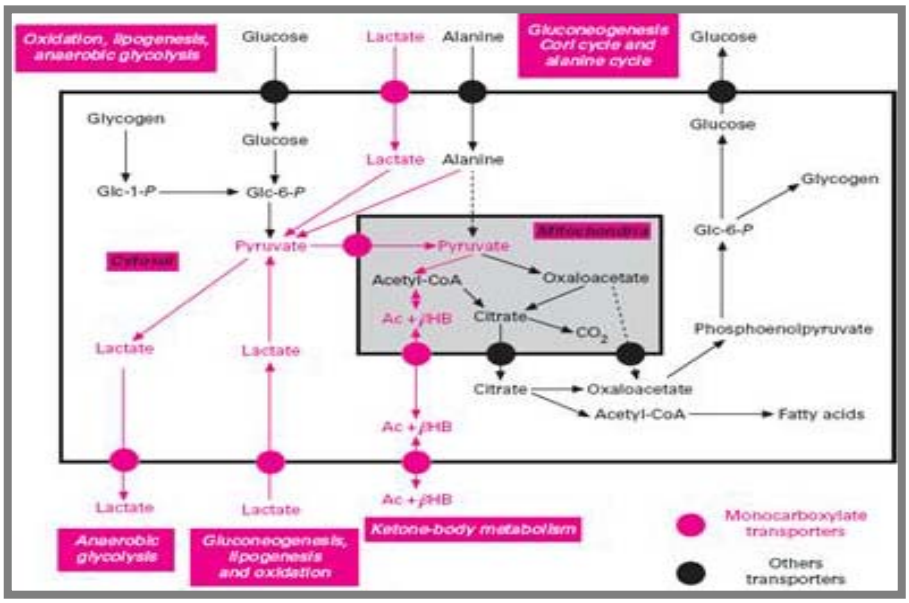

Figure 1: Pathways that involve the transport of monocarboxylates [26].

Fourteen MCTs were already described, each one with properties connected with metabolic requests of the tissues in which they are expressed. Table 1 describes the main characteristics of MCTs family.

Table 1: Principal characteristics of MCT family [32].

The monocarboxylate transporter family			
Protein Name	Predominant Substrates	Transport type/ Coupling ions	Tissue distribution and cellular/ sub cellular expression
MCT-1	Lactate, pyruvate, ketone bodies	C / H^+ or E / monocarboxylate	Ubiquitous
MCT-2	Pyruvate, lactate, ketone bodies	C / H^+	Kidney, brain
MCT-3	Lactate	C / H^+ (pH dependent but cotransport not confirmed experimentally)	Retinal pigment epithelium, choroid plexus
MCT-4	Lactate, pyruvate, ketone bodies	C / H^+	Skeletal muscle, chondrocytes, leukocytes, testis, lung, placenta, heart
MCT-5		O	Brain, muscle, liver, kidney, lung, ovary, placenta, heart
MCT-6		O	Kidney, muscle, brain, heart, pancreas, prostate, lung, placenta
MCT-7		O	Brain, pancreas, muscle
MCT-8	T3, T4 (unpublished)	F	Liver, heart, brain, thymus, intestine, ovary, prostate, pancreas, placenta
MCT-9		O	Endometrium, testis, ovary, breast, brain, kidney, adrenal, retina
TAT1/ MCT-10		Aromatic amino acids (W, Y, F, L-Dopa)	Kidney, intestine, muscle, placenta, heart
MCT-11		O	Skin, lung, ovary, breast, lung, pancreas, retinal pigment epithelium, choroid plexus
MCT-12		O	Kidney
MCT-13		O	Breast, bone marrow stem cells
MCT-14		O	Brain, heart, ovary, breast, lung, pancreas retinal pigment epithelium, choroid plexus
C: Cotransporter E: Exchanger		F: Facilitated transporter O: Orphan transporter	

It is described that the topology of MCTs consists of 12 transmembrane α-helical (TM) domains for MCT1, MCT2, MCT3, MCT7 and MCT8 and between 10 and 12 for the other MCTs, including MCT4. Thus, it seems probable that there are 12 TM domains with the N- and C-termini located within the cytoplasm as it can be seen in Figure 2 [26, 33].

Only the first four members, MCT1-MCT4, have been experimentally demonstrated to catalyze proton coupled transport of monocarvoxylates [26, 34-38] Therefore, they will be the ones to be boarded along this work.

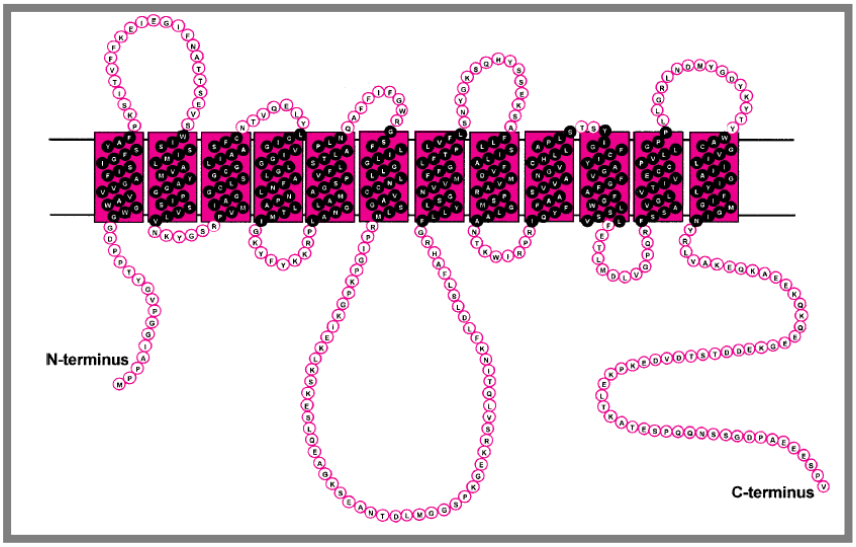

Figure 2: Membrane topology of the MCT family [26]. The sequence showed is of MCT1.

The first MCT, named MCT1, was cloned from Chinese hamster ovary cells and later functionally expressed in a breast tumour cell line. Similar transporters from human, rat and mouse have been cloned and sequenced. Western and Northern blotting has shown that MCT1 is found in the majority of tissues examined in both hamster and rat. MCT1 is encoded by the gene *mct1* and it has been mapped and located in the chromosomal band 1p13.2-p12. It is the most well-studied and functionally characterized member of the MCT family, largely due to the fact that it is the only monocarboxylate transporter expressed in human red blood cells, and it also has the widest tissue distribution. It has values of Km of 1-10mM for the pyruvate, lactate and ketone bodies [20, 33, 39]. Garcia et al [40] have cloned and sequenced the second isoform of MCT from Syrian hamster liver, a tissue where MCT1 is absent. Named MCT2, it shares 60% identity with MCT1 [32, 39]. MCT2 is encoded by the gene *slc16a7* and it is located in the locus 12q14.1. It has great affinity for monocarboxylates, increasing the affinity for the substrate relative to

MCT1. It is present in the cells where a rapid uptake of monocarboxylates is necessary, where these are in low concentrations and in cells which release of the lactate is rare. Thus, it is expressed in proximal kidney tubules, neurons, sperm tails, cardiac myocytes and liver [20, 40, 41].

MCT3 was identified in the retinal epithelium cells of chicken. It is encoded by the gene *slc16a8* and it is located in the locus 22q13.1. It seems to be a very specialized MCT since it is only found on the basal membrane of the retinal pigment epithelium (RPE) of the eye and on the Choroid Plexus epithelium [41-43].

MCT4 is encoded by the gene *slc16a3* and it is situated in the locus 17q25.3. It is a transporter of low affinity adapted for the export of lactate of glycolitic cells like muscle cells, tumour cells and white cells. It is expressed to chondrocytes, leucocytes, testicle, lung and in placenta where is necessary a quick transport of lactate from the fetus to the motherly circulation. The predominant substrates transported for MCT4 are lactate, pyruvate and ketone bodies [20, 41, 44].

To facilitate the interpretation, the following nomenclature regarding genes names will be adopted: for *slc16a1* we will use *mct1*, for *slc16a7 mct2,* for *slc16a8 mct3* and for *slc16a3 mct4.*

Regulation of MCTs

Many membrane proteins are tightly associated with other glycosylated membrane proteins. MCT1, MCT3 and MCT4 have been shown to interact with a cell surface glycoprotein CD147 [45-47] and MCT2 with a closely related protein gp70 [48].

The glycoprotein of cellular surface CD147 also known as basigin or EMMPRIN is widely distributed and expressed in high levels in many tissues, mainly in metabolically active cells such as lymphoblasts and malignant tumor cells. Elevated CD147 stimulates matrix metalloproteinase (MMP)-production in stromal fibroblasts and endothelial cells, leading to extracellular matrix degradation, tumor growth promotion, and metastasis. CD147 also stimulates expression of VEGF [49]. CD147 has an extracellular region with two Ig domains and a N-terminal with several enzyme catalytic sites. The intracellular domain is well conserved between species. The transmembrane domain of CD147 contains a glutamic acid residue, a charged residue which could be involved in the protein association to the plasma membrane (Figure 3). Acting as an essential chaperone, CD147 leads the correct activity and targeting of MCTs to the plasma membrane [47]. MCT2 interacts with an ancillary protein called embigin (gp70), which is a CD147 homologue

with an unreactive Ig-like V domain in place of the C2 domain [46]. Other basigin family members may bind different ligands through the extracellular Ig-like domains, which are less similar than the transmembrane and cytoplasmatic regions [48]. GP70, that shares the ability of CD147 to also interact with MCT1, is strongly and broadly expressed during early stages of embryogenesis, but has a much more restricted distribution in adults [45].

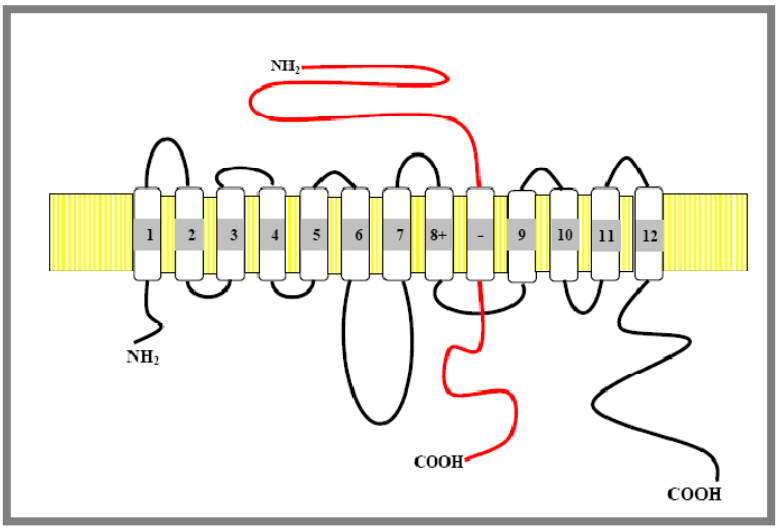

Figure 3: Interaction between MCTs and the chaperone CD147 (red) [41]

MCT Inhibition

There are some chemicals that are known to inhibit the function of MCTs [50]. Several studies have demonstrated the affinity and specificity of each inhibitor to each MCT isoform.

The inhibitors known are [32, 50]:

- Aromatic compounds such as α-cyano-4-hydroxycinnamate (CHC) and phenypyruvate. These are competitive inhibitors and have ki value of 50-500 μM. These inhibitors inhibit MCTs with higher affinity to MCT1, althouth, also inhibit others membrane transporters;
- Thiol reagents as p-chloromercuribenzenesulfonic acid (p-CMBS);
- Stilbenedisulphonates as 4,4'-dibenzamidostilbene - 2,2'disulfonate (DIDS). These inhibitors act as irreversible inhibitors, reflecting covalent modification of the transport. DIDS inhibits MCTs and also inhibits others anion transporters;
- Bioflavonoids such as phloretin and quercetin;

Table 2: Sensitivity of MCT1, MCT2 and MCT4 to each type of inhibitor.

Transporter	Inhibitor	Ref.
MCT1	Sensitive to CHC, PCMBS, DIDS and phloretin;	12,24
MCT2	Sensitive to CHC and DIDS;	12
MCT4	Sensitive to CHC and pCMBS.	12,24

Role of MCTs during embryo development

Studies have shown that embryonic and fetal development are influenced by the oxygen environment and may primarily rely on glycolytic metabolism for its energy supply, a necessary adaptation for the environmental hypoxia that exists during early embryogenesis [4].

Several studies have been already demonstrated the expression of MCTs during pre-implantation development of the human and mouse. The authors [51, 52] demonstrated that since the development of the zygote until the stage of 2 cells, there is an absolute requirement for pyruvate and that lactate could support the development of the embryo in stages of 4 and 8 cells. For that is required a system that is capable of transporting lactate, pyruvate and protons in order to counteract the decreasing of pH inside the cell. Thus, Hérubel et al [20] analyzed and compared the expression of MCT1, MCT2, MCT3, MCT4 and basigin during stages of the early embryonic development of the human and mouse. For both human and mouse the transcript of MCT1 and MCT2 were detected during all stages studied. MCT3 was detected neither in human nor in mouse and MCT4 was detected during all stages but only for mouse. Basigin was also detected in both organisms in all stages.

Another study done during pre implantation development of the mouse embryo was performed by Harding et al [52] that examined changes in the activity of the MCTs during early development and the relationship between MCTs and pHi. They demonstrated the presence of mRNA for MCT1, MCT2 and MCT3 isoforms throughout pre-implantation of the mouse. Furthermore, they studied MCT activity by inhibition with pCMBS and they suggested that MCT1 is the predominant isoform on the regulation of the pHi during early development of the mouse.

AIMS

The purpose of this work was to study the role of MCTs during embryonic development of *Rattus norvegicus* and *Gallus gallus*.

Regarding the study of lung embryo development of *Rattus norvegicus,* the specific aims were:

1. Characterization of MCT1, MCT2 and MCT4 Immunohistochemical expression in different gestational ages of lung development using paraffin sections of fetuses of 13.5, 15.5, 17.5, 19.5 and 21.5 dpc and by Western Blot using fresh tissue samples of the same stages;
2. Evaluation of the effects of MCT inhibition on lung viability and branching (Morphometric analysis) by CHC in fetal lung explants.

Regarding to the chick (*Gallus gallus)* embryo the specific aims were:

1. Characterization of the expression pattern of MCT1, MCT2, MCT3, MCT4 and Hif1α by *In Situ* Hybridization.

CHAPTER 2 – CHARACTERIZATION OF MCTS EXPRESSION PATTERN DURING CHICK EMBRYO DEVELOPMENT

Gallus gallus

The chicken embryo has long been one of the most widely used laboratory animals for both teaching and research and the reasons are clear to see. The chick embryo is well characterized and has been used for the study of embryonic development and analysis of expression patterns of several genes [53]. The advantages of using this model are that fertilized eggs are cheap and available in large number, some genes and physiology of the embryo are well known and the development lasts approximately 21 days [54]. However, the developmental rate can be affected by several factors like incubation temperature, the delay between laying and lab-induced incubation and the season of the year. Thus, a standardized system is needed to characterize the embryo. The Hamburger-Hamilton (HH) system allows the developing chick to be accurately staged both at embryonic and fetal stages, and is used universally in chick embryology (Table 3) [54, 55]. Chick embryos can be "staged" according to different morphological characteristics. In the very early embryo, the primitive streak is the only visible feature, and its shape and size are used to stage HH1-5 embryos. Stages 5-8 may be defined by the formation of the head fold, the neural folds, and their fusion to form the neural tube. The expansion of anterior neural tube to form the brain may also be used to identify later stages. Somitogenesis, the progressive segmentation of the paraxial mesoderm, provides a convenient method for staging embryos between stage 7 and 14. Somites form with surprising regularity every 90 minutes. Stage 10 embryos have 10 somites, and the embryo gains 3 somites during each stage (i.e. Stage 11 embryos have 13 somites; Stage 12 embryos have 16, etc). Formation of the branchial arches, which will give rise to the jaw, pharynx and larynx structures, begins at HH14 and is used as a marker throughout development. The morphology of the limbs, starting with the appearance of wing bud at stage 16, is a useful characteristic for staging chick embryos and fetuses until hatching. Between stages 24 and 35, the appearance of specific structures within the limbs (such as joints and digits); at later stages the length of the toes is used. The formation and development of the eyelids, primordial feathers and beak are used in a similar way to stage later development [1, 54, 55].

Table 3: Chick embryo developmental stages, according to Hamburger & Hamilton (adapted from [55]).

Hamburger Hamilton Stages	Incubation time	Identification of Stages
…	…	…
St4	18-19 hr	Definitive primitive streak,
St5	19-22 hr	Regression of Hensen's node
St6	23-25 hr	Head fold
St7	23-26 hr	1 somite; neural folds
St8	26-29 hr	4 Somites
St9	29-33 hr	7 Somites
St10	33-38 hr	10 Somites
St11	40-45 hr	13 Somites
St12	45-49hr	16 Somites
St13	48-52 hr	19 Somites
St14	50-53 hr	22 Somites
St15-16	50-56 hr	24-28 Somites
St20	70-72 hr	Characterization of limb buds
St25	4 days	Characterization of limb buds

In what concerns to the expression of MCTs during chick embryo development, few studies exist. Han *et al* [4] localized a member of the MCT family, MCT-4, and the chaperone 5A11/Basigin immunohistochemically during early cardiomyocyte differentiation. These results were confirmed by RT-PCR for MCT1, MCT4 and 5A11/basigin. This study was done from stages 5 to 42 of chick embryo development. Relatively to MCT1 expression, it was first detectable at stage 18 and remained expressed throughout the late fetal stages. MCT4 was expressed in all stages studied. Although the band was weak, 5A11 was also expressed in all stages. Another study regarding MCT expression during chick embryo development was performed by Philp *et al* [42] where they described the expression of MCT3. In this study they showed that MCT3 expression is restricted to the membrane of RPE and that MCT3 is firstly detected at embryonic day 5 and persists until the last days of the gestation.

Tissue hypoxia may induce a lot of responses, many of which are dependent on hypoxia-inducible transcription factors (HIFs) [11]. Ullah *et al* [56] demonstrated that MCT4, like others glycolytic enzymes, is up-regulated by hypoxia through a mechanism mediated by Hif1α.

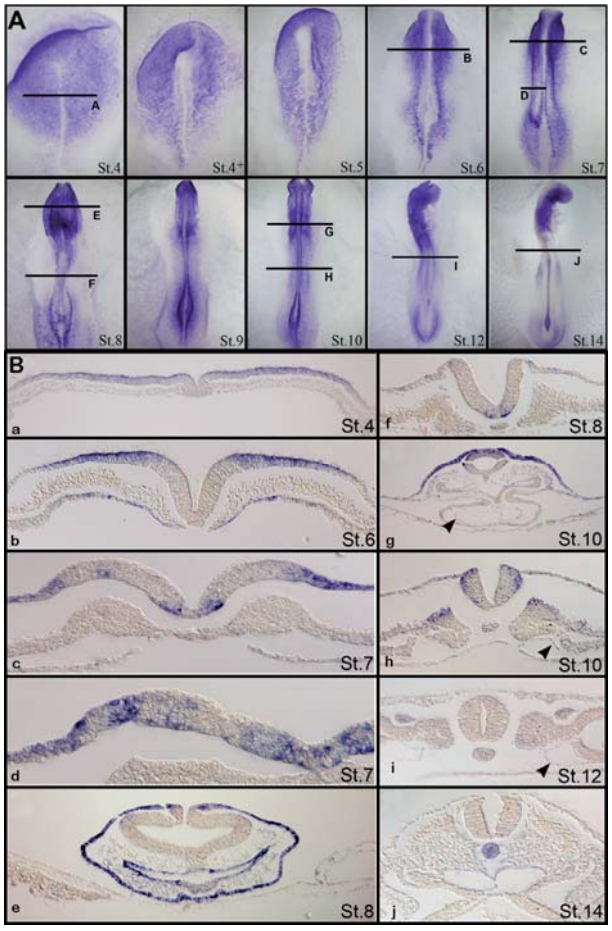

Figure 4: *hif1α* expression pattern in chick embryos from HH4 to HH14, evaluated by whole mount *in situ* hybridization (A) Transversal serial sections of *hif1α* expression (B) [57].

Regarding chick embryo development, there is a study [57] that describes the expression pattern of *hif1α* from stages HH4 to HH14 (Figure 4). They demonstrated that *hif1α* is expressed exclusively in embryonic regions, and was detected mainly in the neural ectoderm at stHH4, on the dorsal neural plate at stHH6 and on restrictive groups of differentiating neurons in closing neural tube. They showed that embryonic non-neural ectoderm and foregut endoderm were also positive for *hif1α*.

Due to the lack of studies relating MCTs and embryogenesis and since this is a complex process that should be explored and understood, we aimed to study the expression of MCTs during chick embryo development and compare the expression pattern of MCTs, mainly MCT4 with the expression pattern of Hif1α.

Material and Methods

1. Eggs and embryos

Chick embryos (*Gallus gallus*) were used for this study. Fertilized eggs were obtained commercially and incubated at 37ºC in a 49% humidified atmosphere. Embryos were staged according to Hamburger and Hamilton (HH) classification [55]. Chick embryos from stage HH4 - HH25 were used in this study.

2. *In situ* Hibridization

In situ hybridization (ISH) is a technique which allows the detection of the spatial distribution of a specific mRNA species in cells/tissues/organs, using an antisense RNA probe. In whole-mount *in situ* hybridization, the entire embryo can be stained for specific mRNAs. This method allows the visualization of the pattern of gene expression in an entire organism [58]. One of the first steps of this technique is the synthesis of the probe to be used. Probes are prepared by *in vitro* transcription using the corresponding cDNA sequences cloned into appropriate plasmids as templates [58].

2.1 Primer design and RT-PCR

To generate *in situ* hybridization probes for MCT1, MCT2, MCT3, MCT4 and Hif1α, primers were designed considering the GenBank gene sequences NM_001006323 (MCT1), XM_416057 (MCT2), NM_205140 (MCT3), NM_204663 (MCT4) and NM_204297 (Hif1α) (Table 4) and RT-PCR reactions were performed.

Total RNA was isolated from whole HH24 and HH34 (2 and 8 days of incubation respectively) embryos. After being harvested, the embryo was immersed and macerated in 2 ml Trizol until an homogeneous mixture was obtained. After homogenization, the sample was left for 5 min at room temperature (RT) to allow complete dissociation of protein complexes and their sedimentation. Then, 0.2 ml of chloroform/1ml of Trizol was added and shaked vigorously. After centrifugation at 13.000 rpm for 15 minutes at 4 ºC, the aqueous supernatant was transferred to a new tube for RNA precipitation with 0.5 ml of isopropyl alcohol, followed by centrifugation at 13.000 rpm for 10 min at 4 ºC. The supernatant was removed and the pellet was washed with 75% ethanol and further centrifuged at 8.000 rpm for 5 min at 4 ºC. After brief drying, RNA was dissolved in 20 µl RNase-free water. Finally, the RNA solution was quantified by spectrophotometry (NanoDrop Technologies) and stored at -20ºC.

The first-strand reaction was carried out using the SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen). In a PCR tube, 1 µl of RNA, 1 µl of Randon primer and 10 µl of DEPC-water were incubated for 10 min at 70 ºC followed by an incubation 5 min at 4 ºC. The following mixture (8 µl) was added to the tube: 2 µl of PCR buffer 10x, 2 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 2 µl of 0.1 M DTT and 1 µl of RNase out. After incubation for 5 min at 42ºC, 1 µl of Super Script RT enzyme was added and the mix was incubated for 50 min at 42 ºC and then for further 15 min at 70ºC. To complete a reaction 1 µl of RNase H was added and the mix was incubated for 20 min ate 37 ºC. The resulting cDNA was then stored at -20 ºC. PCR was performed with the purpose of assessing the presence of these genes in the chick embryo and to prepare cDNA templates for probe synthesis. PCR was carried out with cDNA from stage HH24, in a total volume of 50 µl with the following composition: 2.5 mM $MgCl_2$, 20 µM of each primer, 10 µM dNTPs (Fermentas), PCR 10x buffer (Fermentas), 2 U Taq DNA polymerase (Fermentas) and 1 µl of cDNA. The primer sequences used are shown in Table 4

Table 4: Primer pairs for the genes under study: sequence, annealing temperatures tested and product size.

Primer		Sequence (5'-3')	Annealing temperatures tested	Product size (bp)
slc16a1	Forward	accgtggaggagctctacttctgc	48ºC, 50ºC, 52ºC,56ºC, 58ºC	709
	Reverse	tgatttacaatggggtgtgccacc		
slc16a7	Forward	gtatggtagccgaccagtgatgat	50ºC	651
	Reverse	tatgtttgctaggccttcaatggg		
slc16a8	Forward	atggggagagctgaccgagaggaa	48ºC, 50ºC, 52ºC,56ºC, 58ºC	906
	Reverse	ctggggcaagtgctgctggagaa		
slc16a3	Forward	ggatttcctccattctgttggcca	50ºC	697
	Reverse	aatggtagctggtctcaagtgggt		
hif1a	Forward	agcctcccatgacgtgcttg	50ºC	678
	Reverse	tctcactggatgaggggagca		
gapdh	Forward	ccggaattcatggtgaaagtcggagtcaacg	50ºC	1000
	Reverse	ccgctcgagtcactccttggatgccatgt		

slc16a1 (MCT1), *slc16a7* (MCT2), *slc16a8* (MCT3), *slc16a3* (MCT4)

Reactions were performed in a termocycler as follows: DNA denaturation step at 96 °C for 3 min, followed by 30 cycles of denaturation at 96 °C for 1 min, annealing at corresponding temperature of each primer for 1 min, and extension at 72 °C for 1 min. At the end, a final extension step of 5 min at 72 °C was performed. An aliquot (5 µl) of each PCR product was separated on a 0.8 % agarose gel, stained with ethidium bromide (EtBr) and gel images were visualized with Alphaimager (Invitrogen).

2.2 DNA cloning, transformation and plasmid isolation

Each DNA fragment obtained previously, was cloned into pCR®II-TOPO® vector (Invitrogen). DNA cloning is a method used for isolating a particular sequence of DNA from a complex mixture of different DNA sequences. In order to clone a fragment of DNA, we need to insert it into a vector. A plasmid vector contains three elements: a cloning site where the foreign DNA fragment can be inserted; a drug-resistance gene, which destroys antibiotics – like ampicillin – to allow selective growth of the host cell; and a replication origin to allow the plasmid to replicate in the host cell [58]. The mixture contained 2 µl of PCR product, 1 µl of the salt solution, 1 µl of the TOPO vector and water to a final volume of 6 µl. After mixing, the mixture was incubated for 5 min at RT and then placed on ice.

Each ligation mixture was then inserted into a host cell – *E. coli* bacteria. These cells had been chemically treated in order to make them permeable to DNA molecules. This process is called transformation. Before incubation (30 min), the cells, mixed with the vector, were subjected to a thermal shock at 42 ºC for 20 seconds. Then they were transferred into 250 µl of medium and placed in an orbital incubator for 1 hour at 37 °C with shaking (225 rpm). Subsequently, the bacteria were plated on LB medium containing the antibiotic ampicillin, X-GAL and IPTG. Thus, only the cells that possess the plasmid containing the ampicillin resistance gene could grow. The cells were allowed to grow and multiply at 37 ºC overnight. Only white, isolated colonies were subcultured in a tube containing 5 ml of LB medium with ampicillin and allowed to grow overnight (orbital incubator, 37 °C, 225 rpm). Plasmids were isolated with the GenElute Plasmid Miniprep Kit [(Sigma) Figure 5], DNA was then quantified by spectrophotometry (NanoDrop Technologies, Inc., USA) and stored at -20 ºC.

Restriction analysis was performed to confirm the presence and correct orientation of the insert. This analysis was done by placing in an Eppendorf tube: 8 µl of

plasmid DNA, 0.5 µl of restriction enzyme, 1.5 µl buffer 10x specific of each enzyme and 5 µl of H_2O. The reaction was carried out overnight at 37 ºC and the product was separated on a 0.8 % agarose gel. Gel image was visualized with Alphaimager (Invitrogen). The restriction enzymes for each plasmid are identified in Table 5

The constructs were confirmed upon sequencing.

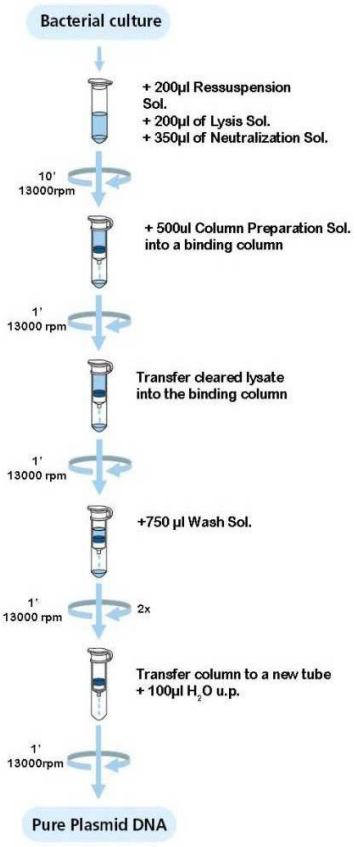

Figure 5: The protocol of the GenElute Plasmid Miniprep Kit used for plasmid isolation.

Table 5: Restriction analyses for confirmation of the insertion and orientation of the DNA fragment in the plasmid. Restriction enzymes and expected fragment sizes for MCT1, MCT2, MCT3, MCT4 and Hif1α plasmids.

Plasmid Name	Restriction Enzyme	SP6 -> T7 (bp)	T7 -> SP6 (bp)
pCRII-MCT1pb	BamH I	3993	4582
		689	100
pCRII-MCT2pb	Nco I	2592	2831
		2032	1793
pCRII-MCT3pb	Pst I	3541	2821
		1190	1190
		82	786
		66	82
pCRII-MCT4pb	Nco I	2500	2969
		2170	1701
pCRII-Hif1Apb	Xcm I	3018	3669
		1633	982

2.3 Synthesis of antisense RNA probes

Synthesized RNA probes were labelled with digoxigenin, a non-radioactive marker. The labelled nucleotides are incorporated into the nucleic acid molecule by an *in vitro* transcription reaction. Before *in vitro* transcription, however, the plasmid should be linearized by a restriction enzyme in a specific site. Then, the linearized plasmid is incubated with the following reaction mixture: 14 µl H_2O RNase free, 7 µl 5x transcription buffer, 4 µl 0.1M DTT, 2 µl 10x DIG RNA labeling mix (Roche), 2 µl RNA polymerase (depending on fragment orientation T7 polymerase or SP6 polymerase) and 2µl RNasin (Promega). After incubation for 3h at 37 ºC, 4 µl of RNase

free DNase (Promega) and 2 µl of RNasin were added and the mixture further incubated at 37 ºC for 30 min. For RNA precipitation 200 µl of TE, 20 µl of 4 M $LiCl_2$ and 600 µl of 100% ethanol were added to the mixture and left overnight at -20 ºC. After centrifugation (12000 rpm at 4 ºC for 30 min), supernatant was discarded and 1 ml of cold 70 % ethanol was added. The supernatant was discarded, the pellet was air dried for a few minutes and ressuspended in 50 µl of H_2O RNase free. The sample was then run on a 0.8 % agarose gel and gel image was visualized with Alphaimager (Invitrogen).

To diminish the size of the probe and to enhance adequate tissue penetration and access to its specific transcript target, a chemical treatment can be done. Thus, the length of the probe was reduced to approximately 200 bases as follows [59]: to 40 µl of labelled RNA probe, 5 µl 0.6 M Na_2CO_3 and 5 µl 0.4 M $NaHCO_3$ were added and the probe was hydrolysed for 30 min at 60 ºC. The time of incubation was determined by the following equation:

$t = (L0 – Lf) / (K \cdot L0 \cdot Lf)$; being:

L0 = starting length of probe RNA (for MCT4 it was 0.7 kb)

Lf = length of probe RNA (in kb) (Lf = 0.2 kb.)

K = rate constant (K = 0.11 kb/min.)

t = hydrolysis time in min

After hydrolysis, the RNA probe was purified with the following solution: 200 µl of H_2O RNase free, 25 µl of 3 M sodium acetate and 600 µl of cold 100 % ethanol, and precipitated overnight at -20 ºC. After centrifugation (12000 rpm at 4 ºC for 30 min), the supernatant was discarded and 1 ml of cold 7 % ethanol was added. After further centrifugation for 15 min, the pellet was left to air dry and resusspended in 80 µl of water. The final RNA probe was stored at -20 ºC.

2.4 *In situ* Hybridization Procedure

All steps of this technique, especially until the second day, must be carried out under RNase free conditions, to protect the mRNA and prevent digestion by ribonucleases. For all reactions, a probe for *fgf8*, previously tested in the laboratory, was used as positive control.

First of all, embryos were rehydrated in a series of methanol/PBT solutions. A series of pretreatment steps before hybridization were performed to increase the efficiency of hybridization and reduce nonspecific background staining [60] In this way and after washing with PBT, the embryos were treated with 20mg/ml proteinase K (pK) to increase the accessibility of the target nucleic acid. The time period of treatment depends on the HH stage of the embryo. Thus for embryos younger than stage HH15, the incubation time in minutes was equal to the HH stage. From stage 16 on, the incubation time was as shown in Table 6:

Table 6: Incubation times of proteinase K (pK) for ISH (embryos from stage 16 on).

HH Stage	Time (min.)	pK
16-18	25	20 mg/ml
19-20	30	20 mg/ml
21-25	35	20 mg/ml
26	40	20 mg/ml
27-29	45	20 mg/ml

After washing with PBT, the embryos were post-fixed in a solution of 37 % formaldehyde and 25 % glutaraldehyde in PBT. After several washes with PBT and hybmix the embryos were incubated overnight with the probe diluted in hybmix (3/1000). Temperature is one of the most important factors that influences the hybridization of the probe to the target mRNA. To find the optimal hybridization conditions, different incubation temperatures were tested.

On the second day, the embryos were passed through a series of washes with Hybmix and MABT. In order to reduce the background, embryos were incubated for more than 1h on a solution of goat serum and blocking in MABT. The embryos were then incubated (overnight) with a solution of goat serum, blocking and MABT containing an antibody against digoxigenin conjugated with alkaline phosphatase (Anti-Digoxigenin-AP, Fab fragments, Roche**).**

On the third day and after washing with MABT and NTMT, embryos were incubated at 37 ºC with a NBT-BCIP solution. This solution is used for the detection of alkaline phosphatase that is conjugated with the antibody. After stained, embryos were photographed at 1-2x magnification (Leica DFC320 digital camera coupled to a Leica MZFLIII stereomicroscope). Some embryos were included in methacrylate and sectioned and slides were visualized under the microscope and photographed.

Results

1. Probe synthesis

In order to study the expression pattern of the genes *mct1, mct2, mct3, mct4 and hif1α* by *in situ* hybridization (ISH), antisense RNA probes for each gene were synthesized. Beforehand, the PCR conditions were optimized to confirm that the primer pairs (Table 4) were able to detect the presence of these genes in chick embryo cDNA.

1.1 PCR optimization

The first PCR reaction was carried out using cDNA synthesized from stage HH24 chick embryo total RNA and all primer pairs, at an annealing temperature of 50 ºC, as described in section 2.1 of Material and Methods. The amplification of a portion of the *gapdh* gene was used as positive control. In these conditions, we only obtained amplification bands with the expected size for *mct2* (MCT2), *mct4* (MCT4) and *hif1α* (HIF-1α) (Figure 6A). For *mct3* (MCT3) we obtained an amplification band under 500 pb, however, the expected size for the amplified product was 906 pb. Thus, and since we did not have amplification for *mct1*, a PCR reaction with a gradient of primer annealing temperature was performed for *mct1* and *mct3*. For this PCR reaction, the same conditions, cDNA and primers pairs for both genes were used as before, but 4 different annealing temperatures were tested for each primer pair: 48 ºC, 52 ºC, 56 ºC and 58ºC. The results are shown in Figure 6B. For *mct1* we obtained amplification bands of the correct size for an annealing temperature of 52 ºC, 56 ºC and 58 ºC. For *mct3*, we obtained the same results as previously (low size of the amplified band). Since this result persisted, even when using another primer pair, we decided to clone and sequence this fragment.

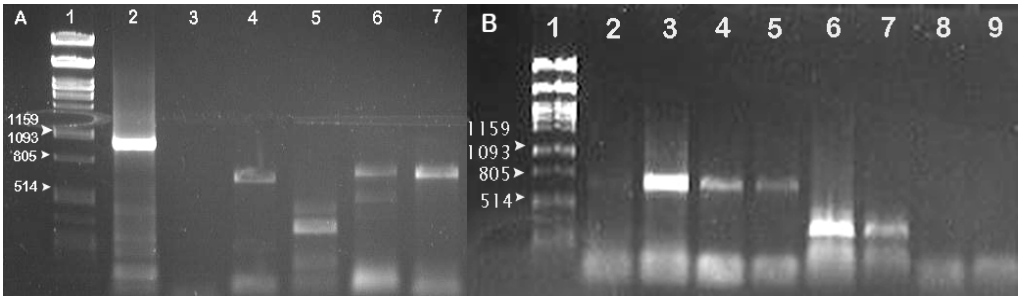

Figure 6: Amplification of a portion of the genes under study by PCR. A: first reaction with an annealing temperature of 50ºC. Lane 1: λDNA digested with *Pst*I used as standard DNA molecular weight marker, 2: positive control, amplification of a portion of *gapdh* of approximately 1000pb, 3: *mct1*, 4: *slc16a7*, 5: *mct3* with an amplification band but with incorrect size, 6: *mct4*, 7: *hif1α*. B: PCR reaction for *mct1* (2-5) and *mct3* (6-9) with temperature gradient. 1: λ*Pst*I, 2,6: 48ºC, 3,7:52ºC, 4,8: 56ºC, 5,9:58ºC.

1.2 Evaluation of insert orientation in the plasmid by restriction analysis

After plasmid isolation using a Miniprep kit (GenElute Plasmid Miniprep Kit, Sigma), and to confirm the presence and orientation of the insert, restriction analyses were performed for each constructed vector. The restriction enzymes used in each case are described in Table 5. For *hif1α*, all candidate vectors were sequenced since no appropriate restriction enzymes could be identified. The restriction fragments obtained for plasmids carrying *mct3* and *mct1* amplicons are shown in Figure 7A and for *mct2* and *mct4* plasmids in Figure 7B. Two possible results were expected for each plasmid depending on the orientation of the insert. By analysis of Figure 7A, we can observe that for *mct3*, we obtained for clones 2, 3 and 5 (lane 2, 3 and 5) 3 bands one of them with approximately 786 pb suggesting that these clones were orientated from T7 to SP6. on the other hand for clone 4 (lane 4) we obtained 2 amplificand (3541 and 1190 pb) suggesting that could be inserted with an orientation from SP6 to T7. Regarding *mct1* restriction analysis for clones 6, 7 and 8 (lane 6, 7 e 8), we obtained a strong amplification band with approximately 4000 pb and a weak band in the region between 514 pb and 805 pb revealing that for those clones the insert could be orientated SP6 to T7. On the other hand, for clone 9 (lane 9) the result obtained is similar to the one that indicate the orientation from T7 to SP6.

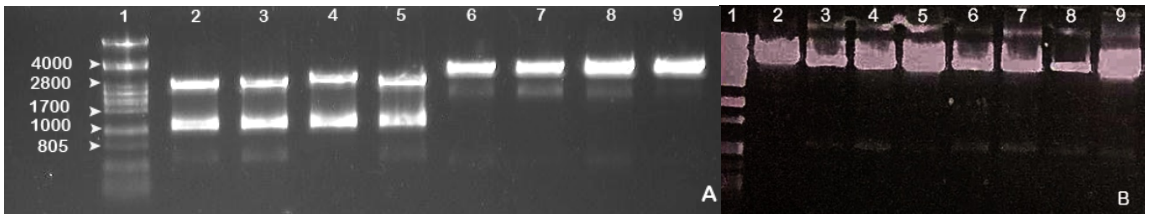

Figure 7: A Restriction fragments obtained for plasmids carrying inserts for **A** - *mct3*, clones 1-4 (lanes 2-5) or *mct1*, clones 1-4 (lanes 6-9); **B**- *mct4*, clones 1-4 (lanes 2-5) or *scl16a7*, clones 1-4 (lanes 6-9) inserts. Lane 1: λ*Pst*I.

Regarding *mct4,* only one clone appear to have a result similar to the expected with two amplified bands with 2500 bp and 2170 bp (Figure 7B, clone 2 lane). For *mct2* only clone 9 (lane 9) revealed a similar result to the expected one (2592 bp and 2170 bp). All these possible clones were sequencing.

After sequencing, the results revealed that, only the plasmids with *mct4* (clone 2) and *hif1α* had the correct insert. *mct4* and *hif1α* were oriented from SP6 to T7, indicating that the synthesis of an antisense RNA probe should be performed using the T7 polymerase.

Figure 8 presents a scheme of the plasmid obtained with an insert for MCT4.

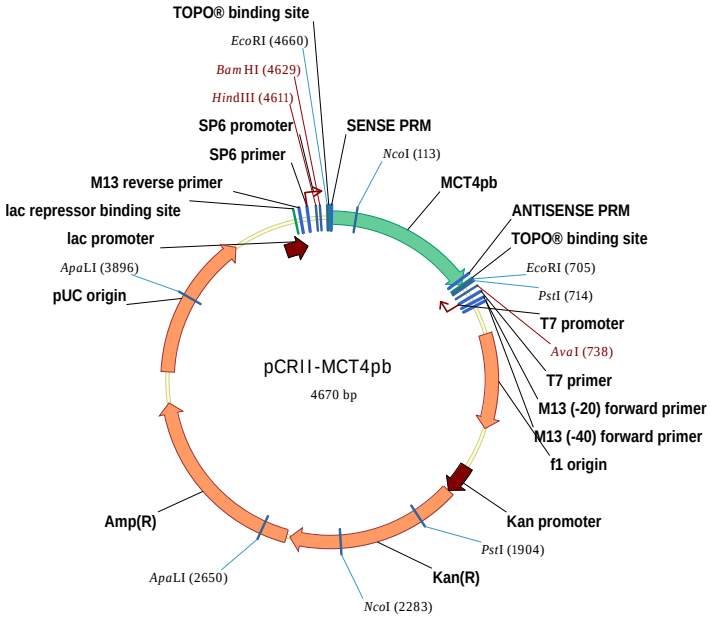

Figure 8: The MCT4 probe plasmid, showing the cloning site where DNA fragment of *MCT4* is inserted (green); the drug-resistance gene (Amp(R)), the replication origin to allow the plasmid to replicate in the host (pUC origin) and SP6 and T7 promoters that allows *in vitro* transcription of sense RNA and antisense RNA respectively.

1.3 Synthesis of antisense RNA probes for *in situ* hybridization

An *in vitro* transcription reaction was performed for both genes to produce an antisense RNA, complementary to the mRNA present in embryo cells, as described in section 0. An antisense RNA probe for *fgf8* was used as positive control of in situ hybridization technique.

2. Optimization of *in situ* hybridization conditions

In situ hybridization technique was already established in the laboratory however, the results were not satisfactory for the genes under study. Thus, the technique required optimization. The parameters tested were the hybridization temperature (37 ºC, 50 ºC and 70 ºC) and the length of the probe, having hydrolyzed the *mct4* and *hif1α* probes to fragments of 200 pb. Satisfactory results for *mct4* probe were only obtained with a hybridization temperature of 50 ºC Figure 9. For *hif1α*, and since we did not obtain staining in any condition tested, we requested probe plasmid to Ota et al [57] *In situ* hybridization was performed with this *hif1α* probe and the results obtained were in agreement with those previously published by these authors.

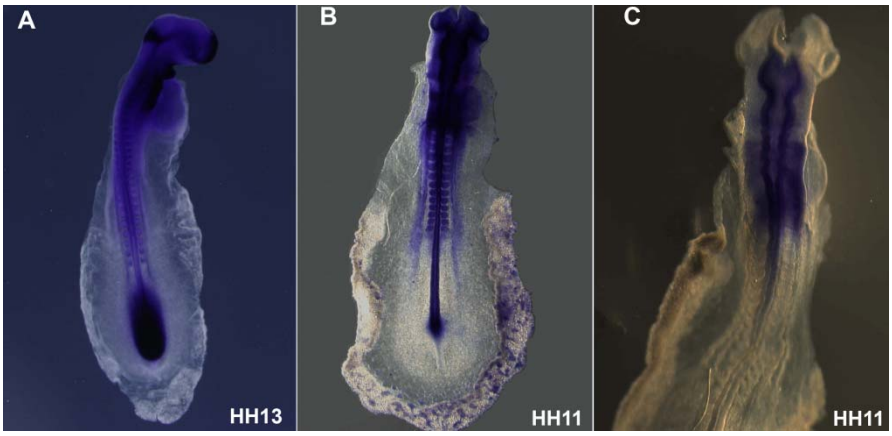

Figure 9: Optimization of *in situ* hybridization conditions. A: positive control – *fgf8* – hybridized at 70ºC; B: hydrolized *mct4* probe hybridized at 50ºC; C: hydrolized mct4 probe hybridized at 70ºC.

3. Expression pattern of mct4 during chick embryo development

The expression pattern of chick *mct4* was characterized for the first time in this study. After preparation of the probe, *in situ h*ybridization experiments were performed in chick embryos from stages HH4 to HH26. By the analysis of Figure 10, we can observe that for all stages studied, *mct4* is expressed in the neural tube and somites, although it is not expressed in early somites, as we can see for stage HH7. By analysis of serial transversal sections of these embryos (Figure 11), we found that neither endoderm nor ectoderm express this gene. We also noticed that *mct4* seems to be mainly expressed in the anterior two thirds of the embryo before stage HH20.

At stage HH7, *mct4* is expressed in the neural fold (Figure 11, section b and c) and open neural plate (Figure 11, section d and e). Expression of *mct4* is not observed in early somites or in either endoderm or ectoderm.

Figure 12 shows stage HH10 (Figure11A) and HH11 (Figure 12C) embryos hybridized with *mct4* probe, and some transverse serial sections from each embryo. At stage HH10, *mct4* expression is detected on the developing head of the embryo, being this expression confirmed on section a and b of Figure 12B. *mct4* is expressed throughout the neural tube and in the somites (Figure 12, section c and d) and is absent from the PSM (Figure 12, section e) and notochord (Figure 12, section d and e). By this stage, *mct4* is expressed in the heart. For stage HH11, *mct4* has a pattern similar to stage HH10. The expression of *mct4* is also detected in the lateral plate mesoderm and it can be observed for both stages (Figure11B, section c and d section h and j). At both stages *mct4* expression is not detected either in the endoderm or in the ectoderm.

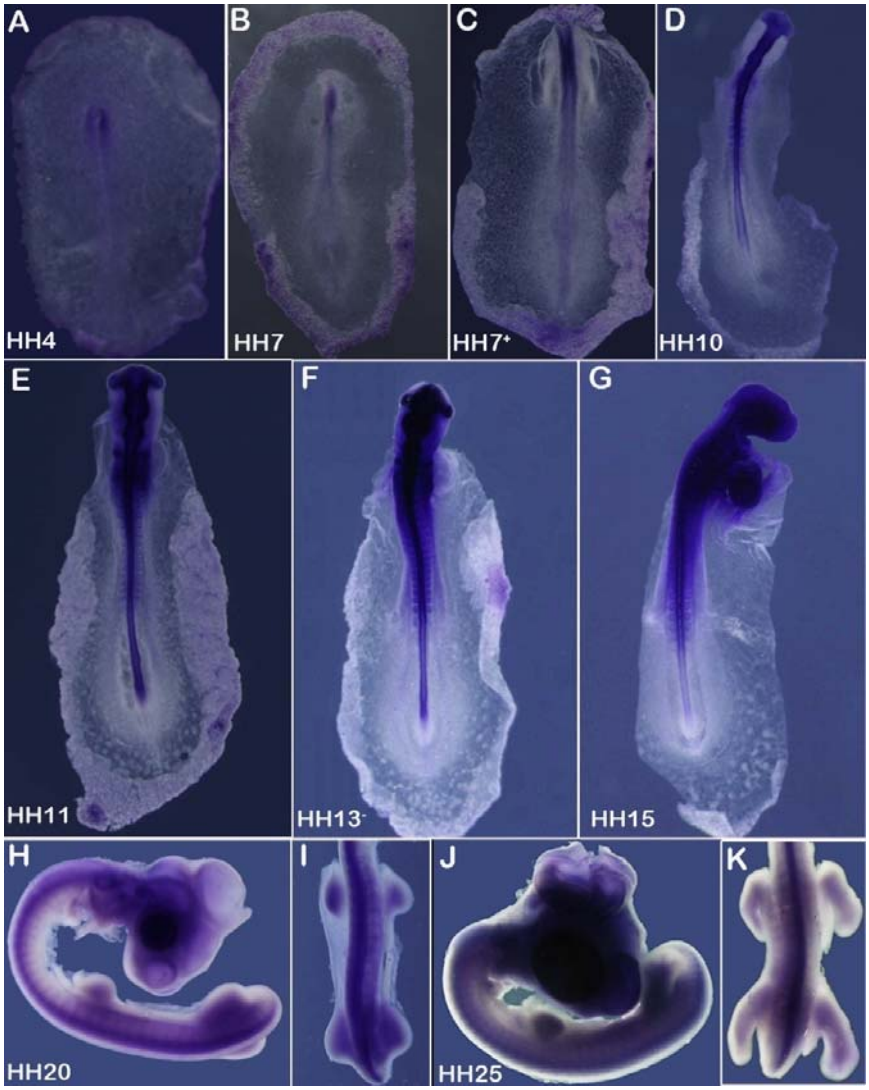

Figure 10: *mct4* expression pattern in chick embryos from stage HH4 to HH25, evaluated by whole mount *in situ* hybridization. All figures present dorsal view, except for H and J: lateral view.

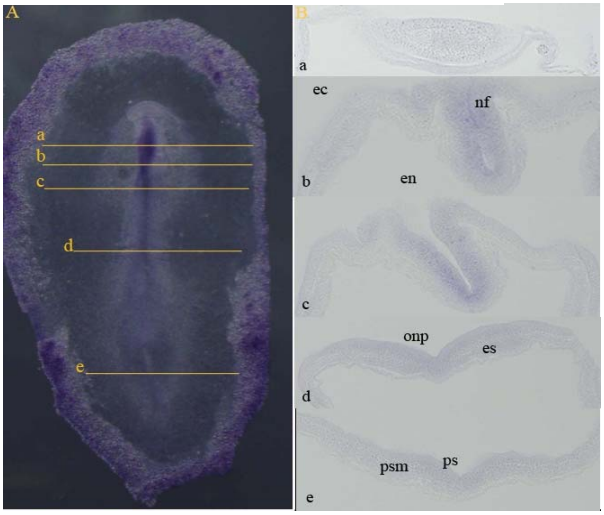

Figure 11: Details of *mct4* expression at stage HH7. A: whole mount *in situ* hybridization, B: Transversal serial sections are shown: a- level of head process b and c- neural fold, d- early somites (es), e- primitive streak (ps). Endoderm (en); ectoderm (ec); open neural plate (onp); pre-somitic mesoderm (psm).

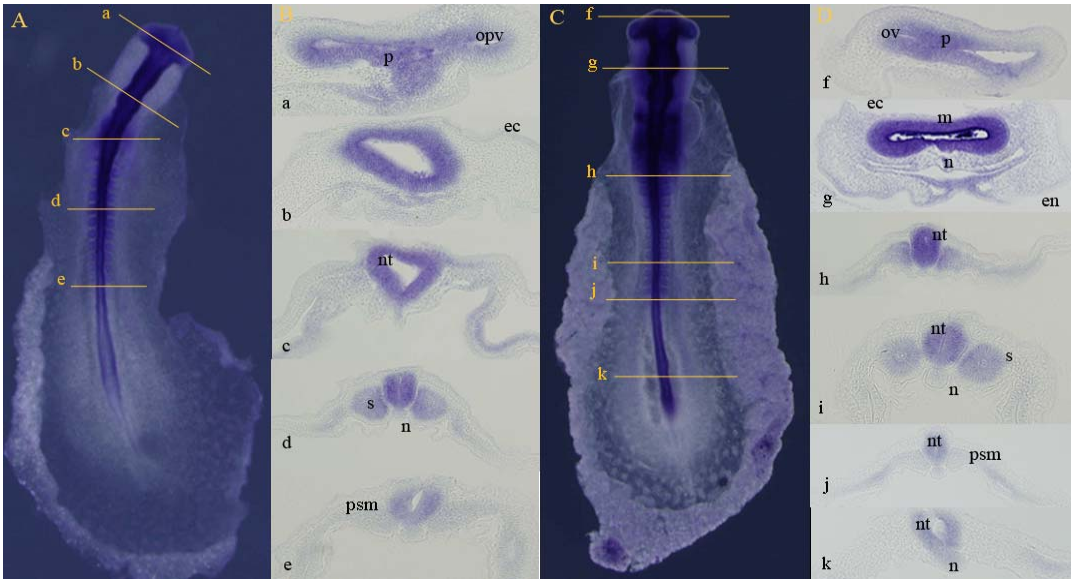

Figure 12: Details of *mct4* expression at stage HH10 (A, B) and stage HH11 (C, D). Transversal serial sections of each embryo are shown (B, D). prosencephalon (p), mesencephalon (m), optic vesicle (opv), neural tube (nt), notochord (n), somites (s),pre-somitic mesoderm (psm), ectoderm (ec), endoderm (en).

For stage HH13^{-} and HH15, *mct4* expression is detected mainly in the anterior part of the embryo. It is expressed in the prosencephalon, mesencephal on and rhombencephalon. Moreover, *mct4* expression is observed throughout the neural tube and in the somites. The lateral plate mesoderm expresses *mct4* as happens for other stages.

In what concerns stage HH20 and HH25, *mct4* expression is observed in the brain (Figure 10). It is expressed throughout the neural tube (Figure 13). On limbs, wing buds and leg buds, *mct4* expression is restricted to mesoderm, being absent from the ectoderm. In stage 25 it appears that *mct4* MCT4 is expressed in the place where digits will be formed (Figure 13B, red arrow).

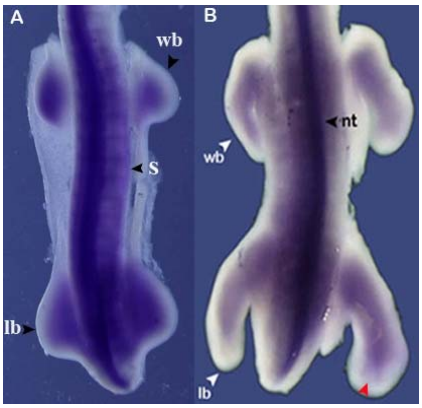

Figure 13: Dorsal view of embryos stage HH20 (A) and HH25 (B), stained by *in situ* hybridization for *mct4*. Wing bud (wb), somite (s), neural tube (nt), leg bud (lb).

Discussion

Embryonic and fetal developmental is strongly influenced by oxygen availability in the environment. Several studies have demonstrated the importance of hypoxia for normal embryonic development in regards to gene expression changes [6]. It is known that cells have capabilities for surviving in hypoxia environments [3]

The expression of *mct4* (MCT4) is detected in all stages of chick embryo development studied. By analyzing our data we observed that *mct4* is mainly expressed in the anterior two thirds of the embryo, in the neural tube, somites and in the developing head. Ullah *et al* [56] showed that MCT4, as the predominant isoform found in glycolytic cells, is over expressed in hypoxia in order to export high quantities of lactate. They also described that this up-regulation of MCT4 expression by hypoxia was mediated by Hif1α. Ota *et al* [57] analyzed *hif1a* expression pattern from stage HH4 to HH14 of chick embryo development by *in situ* hybridization. Comparing their results with *mct4* expression obtained with this study, we can observe that for stage HH4, *mct4* expression does not overlap with *hif1α* expression. At stage HH7, both genes are expressed in the neural fold and in the neural plate, although *mct4* is not found in the lateral plate as *hif1α*. Regarding stage HH10, *mct4* and *hif1α* are both expressed in the head process and along the neural tube.

From our data, it is possible to observe that *mct4* expression is detected in the heart from stage HH10 to HH15. These results are in accordance with previous studies in chick embryo performed by Han *et al* [4]. They demonstrated the expression of MCT4 during cardiac differentiation of chick embryo. Once early development requires hypoxic conditions, they showed by immunohistochemical analyses that MCT4 is expressed within the initial steps of cardiac cell differentiation where EF5, a hypoxia marker, is also expressed. Thus, they conclude that the MCT4, MCT1 and 5A11 expression during chick embryo development is in agreement with the concept that development takes place in a low oxygen environment, and that cardiac cellular energy demands rely on glycolysis. As our data indicate that MCT4 is expressed throughout chick embryo development, and once it was demonstrated that embryos survive in a low oxygen environment, it could mean that cells must have the ability to generate energy by glycolysis during embryogenesis and could use MCT4 for lactate efflux. Unfortunately, we were not able to determine the expression of other MCT isoforms, like MCT1, MCT2 and MCT3. More studies are needed to get these results and to understand the role of MCTs during embryogenesis.

An interesting result is that MCT4 is expressed in the central nervous system structures from the earliest stages, and this expression is maintained in all stages studied. The brain has been shown to express three different MCTs: MCT1, MCT2 and MCT4 [61, 62]. Regarding MCT4 expression it appears to be restricted to astrocytes in adult brain [63, 64]. Rafiki et al [64] demonstrated that MCT4 is present in astrocytes during all stages of development. During development of the brain both lactate and ketone bodies are used as energy substrates [65]. Thus, MCT4 could be exporting lactate that is produced during glycolysis in astrocyte cells. Lactate enters the extracellular space from which it is transported by MCT2 for neuron [61].

To conclude, MCT4 is expressed during chick embryo development, as well as *hif1α*, as previous described by Ota *et al* [57]. The expression patterns of both genes present common domains in some stages, which suggest that MCT4 may play an important role in regulating intracellular pH by the efflux of lactate, since MCT4 is a low affinity transporter involved in monocarboxylate efflux and the embryo development takes place in hypoxic environment, utilizing glycolytic metabolism for energy supply.

CHAPTER 3– ROLE OF MCTS IN LUNG RAT EMBRYO DEVELOPMENT

Rattus norvegicus

Rats are one of the most commonly animals used of all laboratory animals. Due to its extensive use in biomedical research, many biological data about them are currently available. The rat is widely used as an organism model for the study of normal processes and illness in the human, mainly because of a vast body of knowledge of the physiological mechanisms, a significant number of rat models that mimic human disease and because of the easiness of reproduction. Once genes are identified in rats, pathophysiological mechanisms can be elucidated, lending clues to the identification of human genetic counter-parts. The gestation period is only 21 days and litters can number up to fourteen [66]. The development of the lung appears on the day 14^{th} of gestation [1] and starts with the appearance of the tracheal outgrowth from de foregut and ending in early childhood. The bronchial tree develops by branching of the bronchial epithelium around the mesenchyme [67]. The development of the lung can be divided into 5 stages: embryonic, pseudoglandular, canalicular, saccular and alveolar stage .

Embryonic stage starts at 9.5 day pos-conception (dpc) with the budding of primitive lung mass and at 12 dpc lung bud forms the preliminary trachea and pulmonary bronchi forming single left lobe and four lobes of the right lung. Pseudoglandular stage which goes from 13 dpc until 17 dpc is characterized by expansion of bronchial tree, including formation of the bronchi and bronchioles. At this stage epithelia cells start to differentiate to form pre alveolar saccules. In the subsequent canalicular stage (18 dpc-19 dpc) airway branching pattern is completed. During this sage respiratory bronchioli appears, interstitial tissue decreases and vascularization of peripheral mesenchyme increases. During saccular stage (20-22 dpc) the pulmonary parenchyma growth, the connective tissue starts being thin and maturation of the surfactant system occurs. The last stage, alveolar stage (after birth), is characterized by alveoli formation through a septation process [68].

Localized hypoxia is a normal component of embryo development, and it seems to be one of the most important extracellular factor for lung morphogenesis which is a complex process [69, 70]. Troug *et al* [69] evaluated the hypoxia effects on lung development. After exposure of pregnant females for 10 days to hypoxia, rat embryos were harvested with 14 days of gestation. They used the rat-specific wide array chip analysis and detected upregulated and downregulated genes after rat lung hypoxia

exposure. One of the upregulated genes found was MCT4. They concluded that hypoxia interrupted lung development and that several pathways are involved in this mechanism.

Preliminary results obtained in ICVS showed different expression of MCT1, MCT2 and MCT4 throughout the embryonic lung development of *Rattus Norvegicus.* Since there are no studies in the literature on the expression and activity of these molecules during lung embryonic development, in this work we aimed to study and characterize MCT expression in lung development and to evaluate the effect of MCT inhibition on branching morphogenesis of rat fetal lung.

Material and Methods

1. Animals model

Female rats were maintained in appropriate cages under controlled conditions. All pregnant rats were sacrificed by decapitation at 13.5 dpc. After that, fetuses were removed by caesarean section and the dissection of the lungs was gently made by Cristina Silva (MD, PhD student).

2. Fetal lung explants cultures

After harvesting of the lung, they were transferred to Nucleopore Membranes that had been put in DMEM for 1hour in a 24-well culture plates. 200 µL of medium was added to the explants and they were incubated for one hour in 5 % CO_2 at 3 ºC. The composition of the medium consisted of: 50 % of DMEM, 50 % of nutrient mixture F-12 (Invitrogen) with 100 mg/mL of streptomycin, 100 units/mL of penicillin (Invitrogen), 0.25 mg/mL of ascorbic acid (Sigma) and 10 % of fetal calf serum (Invitrogen). After one hour of incubation 2 µl of the inhibitor was added. Fetal lung explants were incubated in a 5 % CO_2 for 96 h at 37 ºC. The medium was replaced every 48 h and the inhibitor – CHC - was daily added. Fetal lungs were photographed daily and D0 was considered the first day and D4 the last. At the end of the incubation time, explants were subjected a series of washes in PBS and were stored at -80 ºC until use.

3. Morphometric analysis of lung explant

The lung explants were daily photographed to monitored branching morphogenesis. The total number of peripheral airway buds (branching) was determined at D0 and D4, such as the area, using AxionVision Rel. 4.3 software. For all experimental conditions, the results of branching and area were expressed as D4/D0 ratio.

4. Immunohistochemistry

Immunohistochemistry (IHC) is a technique that consists on the localization of proteins in a cell or a tissue section. It is based on the linking of a specific antibody (ab) to an antigen (ag) [71]. For IHC studies paraffin sections of fetuses with 13.5, 15.5, 17.5, 19.5 and 21.5 dpc were used to study MCT expression during lung

development. IHC was also performed in lung explants used for the inhibition studies. These lungs explants were fixed in formalin and embedded in paraffin. Sections of 5µm were used for IHC. IHC was performed according to avidin–biotin–peroxidase complex principle (R.T.U. VECTASTAIN Elite ABC Kit (Universal), Vector Laboratories). Paraffin embedded sections were deparaffinized in xylene and hydrated in a graded series of ethanol. During hydratation paraffin sections were treated with 0.3% hydrogen peroxide to inactivate endogenous peroxidases. For antigen retrieval, slides were incubated with 10mM citrate buffered solution (pH 6.0) for 20 min in water bath at 98ºC and then washed in PBS. Slides were incubated with a blocking solution (Normal Horse Serum) for 20 min and then followed by incubation overnight at room temperature with the primary antibody:

- MCT1 (AB3538P,Chemicon) diluted 1:200;
- MCT2 (sc-14926, Santa Cruz Biotechnology) diluted 1:50;
- MCT4 (AB3316P, Chemicon) diluted 1:200.

After rinsing in PBS slides were incubated with a secondary biotinylated antibody for 30 min and then with R.T.U. Vectastain® Elite ABC reagent for 45 min at 37°C. After that, tissues were stained with 3,3′-diamino-benzidine (DAB+ Substrate System, DakoCytomation) for 10 min, counterstained with haematoxylin and mounted with Entellan®.

For positive control normal tissue of skin were used for MCT1 and MCT4 and kidney for MCT2.

The slides were evaluated and then photographed under a microscope.

5. Western Blot

This is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. For the detection of MCT1, MCT2 and MCT4 the antibodies used in immunohistochemistry were evaluated by Western Blotting. The protein was extracted from the lung explants at stage 14. After maceration of the lungs with lysis buffer, the homogenate was centrifuged at 13000 rpm 15 min at 4ºC. The supernatants were collected and protein concentrations were determined by using the Kit *DC* Protein Assay (BioRad). Protein extracted from fetuses lung of stages 15.5, 17.5, 19.5 and 21.5 were already available. Tissue samples of colon were used as positive controls for the expression of MCT1, 2, and 4. 20 ug of protein of each sample were separated on a 10% (w/v) polyacrylamide gel and transferred onto a nitrocellulose

membrane. After that, membranes were blocked with 0.1% Tween, 5% milk in TBS for1h and then incubated overnight at 4°C with primary polyclonal antibodies. After washing with TBS-0.1%Tween, membranes were incubated with the secondary antibody coupled to horseradish peroxidase (SantaCruz Biotechnology). For MCT1 and MCT4, the secondary antibody used was an anti-rabbit antibody with a dilution of 1:10000 in TBS-0.1% Tween, 1% milk. For MCT2 and β-actin an anti-goat antibody with a dilution of 1:5000 in TBS-0.1% Tween, 5% milk was used. At the end, bound antibodies were visualized by chemiluminescence with the SuperSignal West Femto Maximum Sensivity Substrate. The results were quantified by ImageJ Software.

Results

1. Expression of MCTs during lung embryo development

Expression of MCTs was evaluated by Immunohistochemistry and Western Blot. Preliminary results performed in our laboratory had demonstrated the presence of MCTs throughout lung development. In the present study we repeated some immunohistochemical studies with different samples (from other animals) to confirmed that results. Thus, Immunohistochemistry and Western Blot were carried out on samples corresponding to stages 14 (13.5 dpc), 16 (15.5 dpc), 18 (17,5 dpc), 20 (19.5 dpc) and 22 (21.5 dpc) of rat lung development for MCT1, MCT2 and MCT4.

1.1 Expression of MCT1

By analysis of Figure 14 we observed that MCT1 is more expressed at the earliest stage of rat lung development (13.5 dpc) and decreased in subsequent stages being absent during the saccular stage (19.5 dpc) of rat lung development. For stage 14, 16 and 18 staining is cytoplasmatic and occasionally membrane and it is restricted to the bronchial epithelium confined to the bronchial lumen. During stage 22 MCT1 is also expressed in the cytoplasm and membrane of the cells. In this stage, although the staining appeared in the bronchial epithelium, it also appeared in some stromal cells.

Western blot analysis revealed a band around 50 Kda consistent with the molecular weight described for this protein (Figure 15). MCT1 expression was observed in all stages studied. By analysis of Figure 20 it appears that MCT1 expression decreases along lung development.

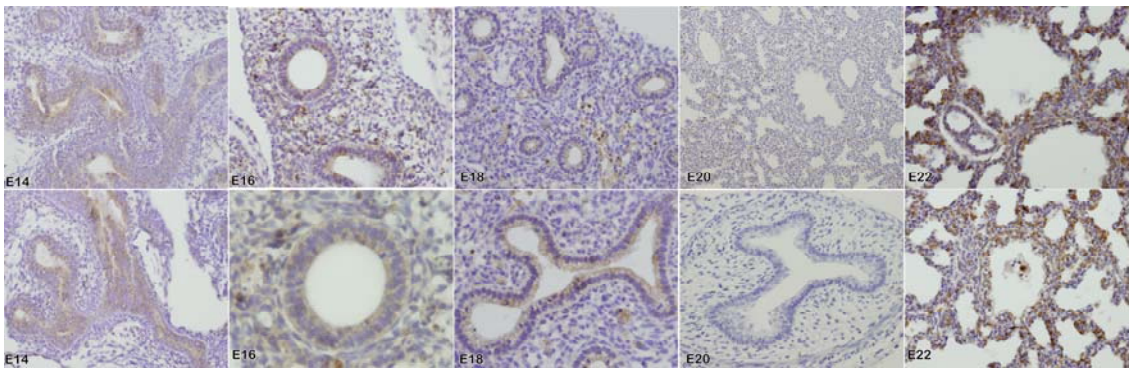

Figure 14: Immunohistochemical expression of MCT1 during normal rat lung development. E14: 13,5 dpc (n=2); E16: 15,5 dpc (n=2); E18: 17,5 dpc (n=2); E20: 19,5 dpc (n=2); E22: 21,5 dpc (n=2).

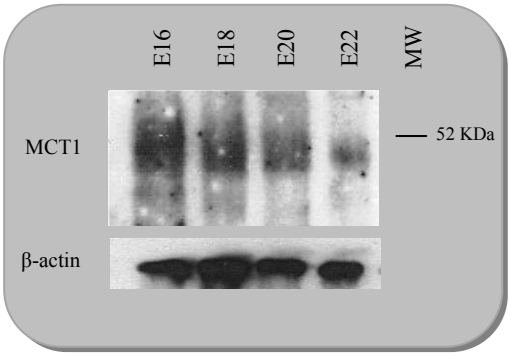

Figure 15: Western blotting for MCT1 in fetal rat lung, E16: 15,5 dpc; E18: 17,5 dpc; E20: 19,5 dpc; E22: 21,5 dpc. The reference gene used was β-actin. MW: molecular weight. –

1.2 Expression of MCT2

IHC studies revealed that MCT2 expression is not detected in the earliest stages of rat lung development, during the pseudoglandular stage (13.5 and 15.5 dpc). But it is expressed in the bronqui epithelial cells of the canalicular stage (17.5 dpc) and it is weakly expressed or even absent in stage 20 (Figure 15). By stage 22, MCT2 it is not expressed in the fetal rat lung. Regarding MCT2 expression during the canalicular stage (17.5 dpc), it has both a cytoplasmatic and membrane staining and as for MCT1 the cytoplasmatic expression is restricted to the bronchial epithelium with stronger staining in the apical region.

For MCT2 we obtained by western blot a band around 50 Kda consistent with the molecular weight described for this protein (Figure 17). As for MCT1, we observed that MCT2 is expressed in all stages studied. Protein levels of MCT2 and β-actin were measured and MCT2 expression seems to increase with lung development (Figure 20).

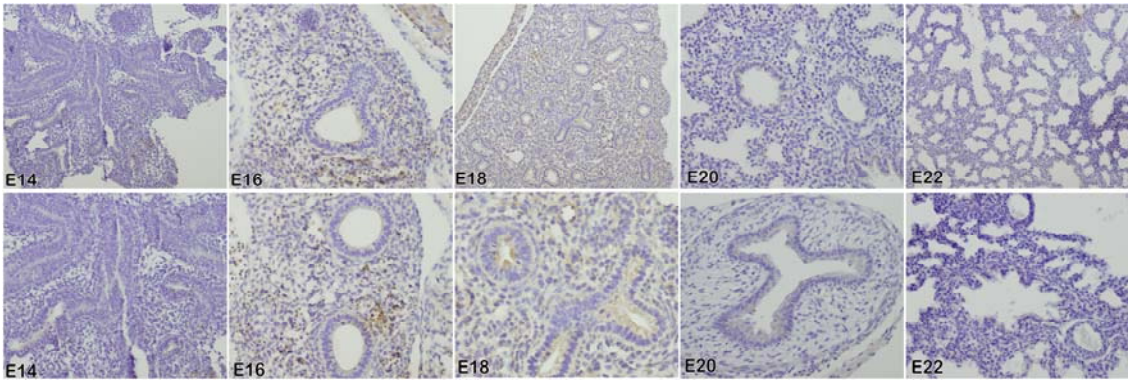

Figure 16: Immunohistochemical expression of MCT2 during normal rat lung development. E14: 13,5 dpc (n=2); E16: 15,5 dpc (n=2); E18: 17,5 dpc (n=2); E20: 19,5 dpc (n=2); E22: 21,5 dpc (n=2).

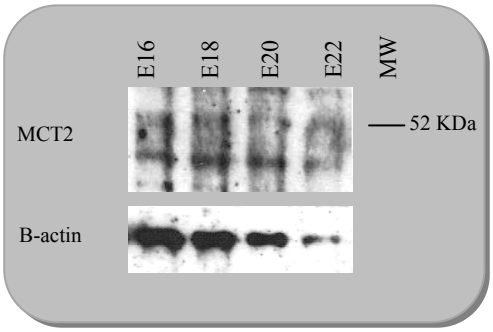

Figure 17: Western blotting for MCT2 in fetal rat lung, E16: 15,5 dpc; E18: 17,5 dpc; E20: 19,5 dpc; E22: 21,5 dpc. The reference gene used was β-actin. MW: molecular weight. ~

1.3 Expression of MCT4

As for the other isoforms, IHC studies revealed that expression of MCT4 varies throughout lung development. MCT4 expression is observed in the earliest stage of rat lung development (13.5 dpc) with a cytoplasmatic and membrane expression in the bronchial epithelium directed to the bronchial lumen. Differently from what happens to the other isoforms, MCT4 is also expressed in the peri-bronchial region. This expression pattern is repeated for stages 18 and 22 although, staining is predominantly in the membrane, with stromal cells also stained. However, during stages 16 and 20 of lung development MCT4 expression is absent (Figure 18).

MCT4 expression was also detected in all stages studied, with protein band around 50 Kda by Western blot (Figure 19). Protein levels of MCT4 and β-actin were measured with IMAGEJ program and as we can observe in Figure 20, MCT4 expression slightly decreases up to stage 20 and increases from stage 20 to stage 22.

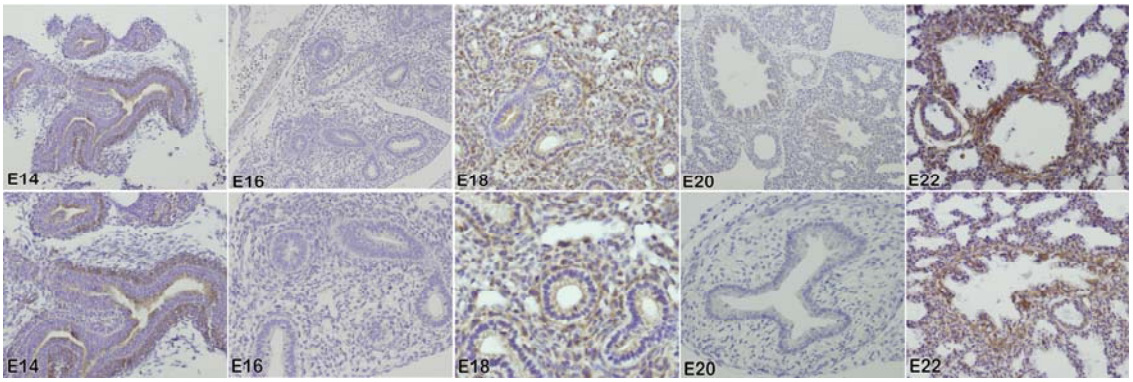

Figure 18: Immunohistochemical expression of MCT4 during normal rat lung development. E14: 13,5 dpc (n=2); E16: 15,5 dpc (n=2); E18: 17,5 dpc (n=2); E20: 19,5 dpc (n=2); E22: 21,5 dpc (n=2).

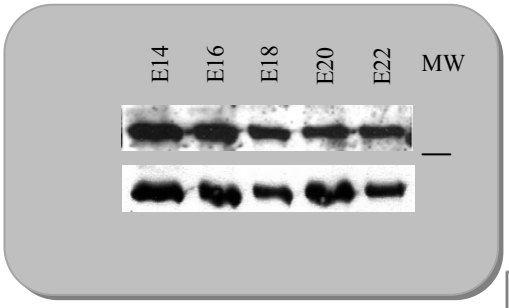

Figure 19: Western blotting for MCT4 in fetal rat lung, E14: 13,5 dpc; E16: 15,5 dpc; E18: 17,5 dpc; E20: 19,5 dpc; E22: 2w1,5 dpc. The reference gene used was β-actin; MW: molecular weight.

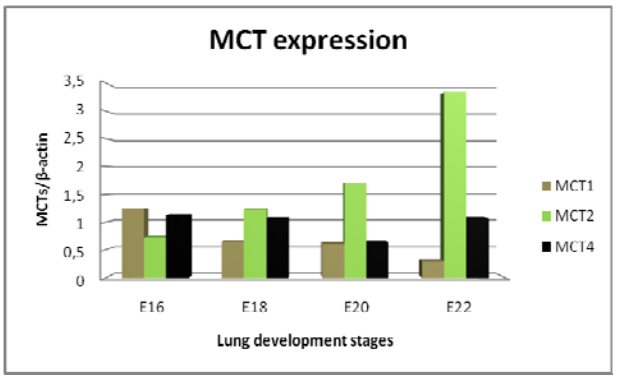

Figure 20: MCT isoform expression throughout lung rat embryo development (E16-E22). Quantification of western blot results of Figs 14,16 e 18. Results are presented as MCT/ß- actin ratio.

Table 7: MCT isoforms expression throughout lung rat embryo development by IHC.

MCT isoform	Lung development stage	IHC expression	Localization
MCT1	E14	+	Cytoplasmatic and membrane; Bronchial epithelium;
	E16	+	´´
	E18	+	´´
	E20	-	
	E22	+	Cytoplasmatic and membrane; Bronchial epithelium; Stromal cells
MCT2	E14	-	
	E16	-	
	E18	+	Cytoplasmatic and membrane; Bronchial epithelium;
	E20	-	
	E22	-	
MCT4	E14	+	Cytoplasmatic and membrane; Peri-bronchial and Bronchial epithelium;
	E16	-	
	E18	+	Cytoplasmatic and membrane; Peri-bronchial and Bronchial epithelium; Stromal cells;
	E20	-	
	E22	+	Cytoplasmatic and membrane; Peri-bronchial and Bronchial epithelium; Stromal cells;
(+) – positive expression; (-) – negative expression			

1. MCT inhibition in lung explants

Lung explants were cultured and treated with CHC to determine which would be the effect of MCT inhibition in branching morphogenesis of the lung. For that it was studied different doses of CHC (0.1mM to 50 mM).

The first experiments were performed with the addition of 2µl of inhibitor directly in explant culture. However, for higher concentrations (10 and 50mM) we observed a pH alteration of the medium (this changed color). In fact after 24h of incubation these lungs showed no viable. In Figure 22, representative examples of fetal lung explants of these experiments are illustrated. Comparing fetal lung explants treated with 0.1mM and 1mM with control (DMSO) at D4, it appears that CHC had an inhibitor effect. On the other hand, as lung explants treated with 10 and 50 mM were not viable at D4 we decided to perform an experiment where the medium pH with the inhibitor was controlled (pH 7.4) before being added to the explants culture. In Figure 22, representative examples of fetal lung explants, with pH controlling, are illustrated. Comparing fetal lung explants treated with CHC at D4 with control (DMSO) it appears that CHC had an inhibitory effect.

Morphometric analysis results, of the fetal lung explants are shown in Figure 22.

CHC appears to have an inhibitor effect in branching morphogenesis of lung development in a dose dependent way. However, the number of lung explants used for each concentration is low. These experiments should be repeated in a higher representative sample to confirm these results.

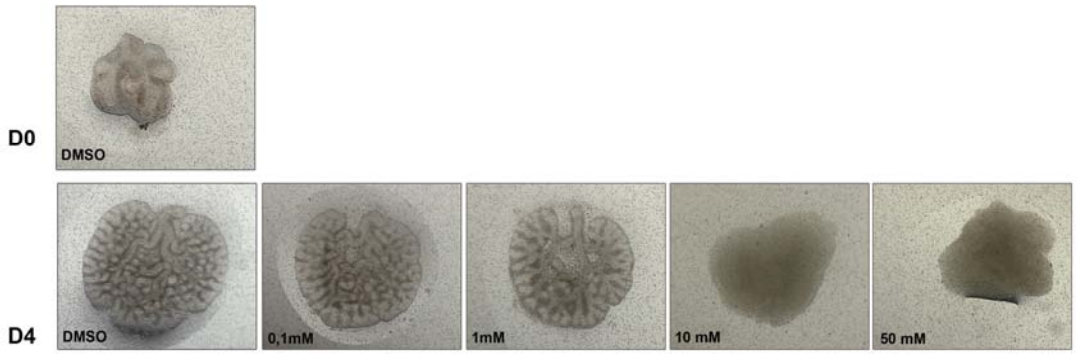

Figure 21: Rat lung explant system treated with increasing concentrations of CHC. Upper image, lung explant with no treatment (control) at D0; bottom lung explants treated with increasing doses of CHC at D4. Experiment with no pH controlling of inhibition solutions.

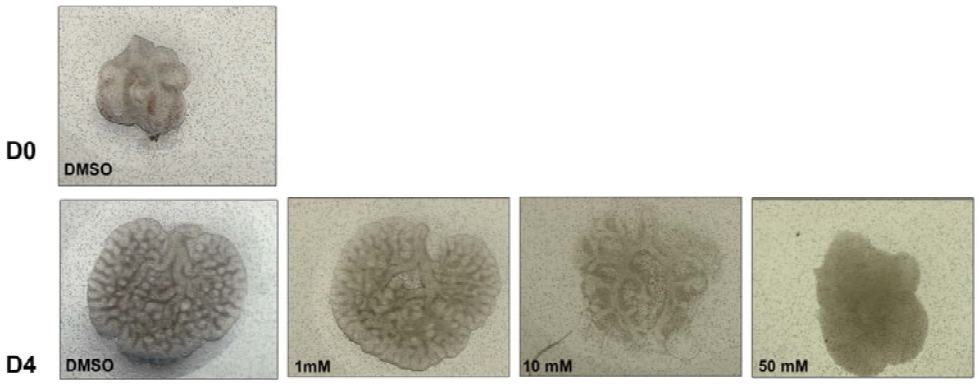

Figure 22: Rat lung explant system treated with increasing concentrations of CHC. Upper image lung explant with no treatment (control) at D0 and at the bottom lung explants treated with increasing doses of CHC at D4. Experiment with pH controlled.

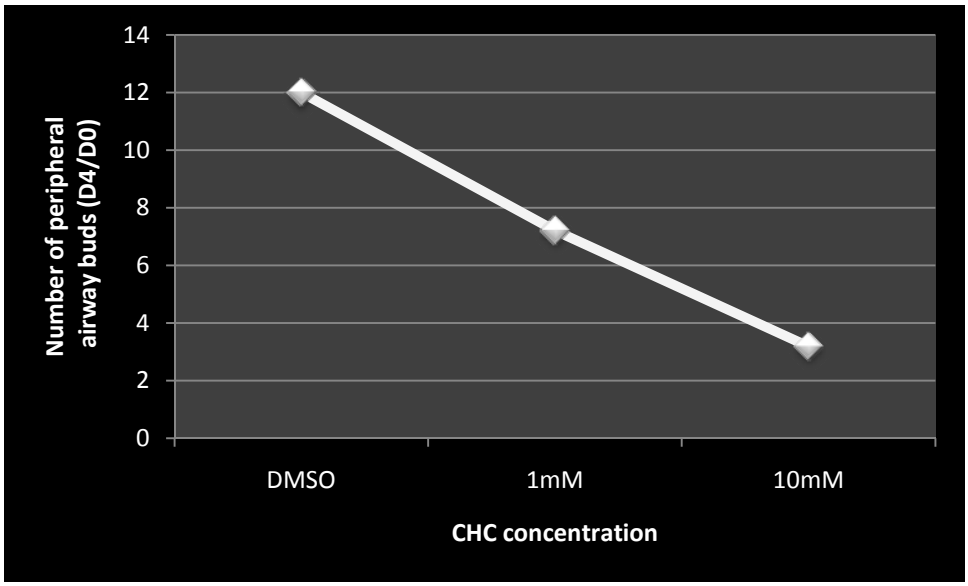

Figure 23: Morphometric branching analysis of rat lung explant system treated with different CHCdoses: total number of peripheral airway buds (D4/D0 ratio). DMSO (n=3); 1mM (n=6); 10mM (n=3)

DISCUSSION

Lung development involves several biochemical, cellular and ultrastructural changes [70] There are no studies on the role of MCTs during lung embryo development. The present study demonstrated that in normal fetal lung, MCT isoform expression varies throughout the lung development stages. MCT1 and MCT4 were expressed in primitive epithelial cells as early as the pseudoglandular stage (13.5 dpc) of lung development while MCT2 was absent on this stage. Stage 14 samples used were lungs harvested from embryos which were cultured for 96 hours and this fact could have interfered with protein expression. Thus, it is important to repeat those studies using freshly harvested lungs from embryo at 13.5 dpc. Regarding stage 16 and 18, MCT1 remained expressed although with a lower intensity. MCT2 was not expressed in stage 16 however, during canalicular stage (17.5 dpc) it was expressed in the cytoplasm and membrane of bronchial epithelium cells. MCT4 was not expressed in stage 16, but its expression was detected during canalicular stage. Curiously, during saccular stage (19.5 dpc) no MCT isoform was expressed, which could be due to the fact that this stage is characterized by alveolar formation. On stage 22, MCT1 and MCT4 were both expressed in the bronchial epithelium cells, in the peri-bronchial region and in the stroma.

Since for western blot samples (homogenized tissue), which contained other cells rather than lung tissue (e.g. erythrocytes) was used, we obtained MCT positivity for stages that were negative by IHC. Besides, we obtained a blot with some bands with different sizes. This could be due to the fact that the antibodies used were described as human specific and only with 60% of homology with rat. Bonen *et al* [72] performed a study where they examined MCT protein expression in a large number of rat adult tissues. They used several antibodies for each MCT isoform, and they used the same antibodies for MCT1 and MCT4 as we used. They conclude that these antibodies detected bands with different sizes in rat tissues (35 KDa for MCT1 and some bands between 35-50 KDa for MCT4) from those obtained in human tissues (46 KDa for MCT1 and 52 KDa for MCT4). However, this report is only based by the visualization of the blot and, in their study, they did not do complementary analysis such as densitometric quantitation, correlation with mRNA and peptide antigen blocking, to validate specificity. Besides, we compared our results with human tissue. Thus,

We considered the band corresponding to the size of our human positive control (around 50 KDa).

Our data demonstrated that the three MCT isoforms are distinctly expressed throughout rat lung embryonic stages development, suggesting that they could be involved in lung development. MCTs have already been identified in other organs during embryo development. Han *et al* [4] demonstrated that MCT4 is expressed during chick cardiac cell development and that MCT4 is crucial for cell development in hypoxic environment of embryogenesis. Troug *et al* [69] showed that after 10 days of exposure to hypoxia, MCT4 was one of the genes upregulated in fetal lung. Thus, MCTs may have an important role during lung embryo development, and could be related with the adaptation of the pulmonary cells to hypoxia.

In order to understand if MCTs played a role in lung morphogenesis, some studies using a fetal lung explant models were performed. Increasing doses of CHC, inhibitor of MCTs, were added to lung explant and it was observed that as CHC concentration increased, the lungs presented higher branching inhibition and with the higher dose (50mM) they were not viable. Also the sample number should be higher. Although CHC is considered as a classical inhibitor for MCT activity, it is not only specific for these transporters and can inhibit others anion exchangers as mitochondrial pyruvate transporter however, there are no evidences showing CHC plasma membrane uptake [73]. Thus, further experiments (e.g. RNAi) should be done to determine the specific role of MCTs on branching morphogenesis in lung development.

CHAPTER 4 – CONCLUSION

CONCLUSION

Embryonic developmental is strongly influenced by oxygen availability in the environment [2]. Several studies have demonstrated the importance of hypoxia for normal embryonic development in regards to gene expression changes [3]. It is known that cells are able to surviving in hypoxia environments using glycolytic metabolism to supply energy. In this study we used two animal models, *Gallus gallus* and *Rattus norvegicus*, where MCT expression was characterized. The expression of *mct4* varies throughout chick embryo development. Regarding rat embryo lung development the expression of MCT1, MCT2 and MCT4 also varies. However, more studies will be need in both organisms to understand the role of MCTs in embryogenesis and to prove embryo dependence on MCT activity.

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